Axial differences in endocytosis along the kidney proximal tubule

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Mini-Review:

Axial differences in endocytosis along the kidney proximal tubule

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Abstract

The proximal tubule (PT) reabsorbs filtered proteins via receptor mediated endocytosis to prevent energetically inefficient wasting in the urine. Recent intravital imaging studies have suggested that protein reabsorption occurs in early (S1) segments, which have a very high capacity. In contrast, uptake of fluid phase substrates also occurs in distal (S2) segments. In this article, we will review these findings and their implications for understanding integrated proximal tubular function, patterns of damage caused by endocytosed toxins, and the origins of proteinuria. We will also discuss whether compensatory downstream increases in protein uptake might occur in disease states, and the environmental factors that could drive these changes.

Introduction

The proximal tubule (PT) is the workhorse of the kidney, carrying out the bulk of reabsorption of fluid filtered by glomeruli. In addition, it specifically reclaims small proteins (including peptide hormones, enzymes and plasma low molecular-weight proteins) to prevent wasting in the urine. This is not only important for energy balance, since amino acids are a crucial metabolic fuel for the body, but also stops potentially harmful bioactive proteins from reaching the distal nephron (33). PT cells have a highly developed endo-lysosomal system and express two large apical receptors (megalin and cubilin), which bind filtered proteins and internalize them via receptor mediated endocytosis (4). Various genetic and acquired insults can adversely affect PT endo-lysosomal function, resulting in increases in urinary protein excretion (so called tubular proteinuria) (3, 11, 13, 18, 43).

The morphology of PT cells has been extensively described (5, 28, 29). Early light and electron microscopy observations established the presence of three consecutive segments (termed S1-S3), which could be distinguished by differences in cellular ultrastructure. Such a segregation was clear in the kidneys of most mammals (including rats, rabbits, dogs and humans), but segmental differences were thought to be less pronounced in mice. Generally speaking, uptake of filtered fluid predominates in the early PT, while secretion of non-filtered substances (e.g. organic anions) is more pronounced in later parts (24, 26, 27, 49). However, the extent to which S1-S3 really represent functionally distinct entities continues to be a matter of debate, especially in mice, which are increasingly prominent in kidney research due to the relative ease of genetic manipulation compared to other species. Moreover, unlike in the distal nephron, the situation in the PT is complicated by a lack of established segment-specific antibody markers. Nevertheless, gene expression studies have confirmed the existence of substantial axial differences along the PT in both mice and rats (6, 20).

Interest in functional heterogeneity along the PT has been re-awakened recently by live imaging studies. For example, substantial differences in metabolic autofluorescence signals have been reported between S1 and S2 segments using multiphoton microscopy (2, 14). In

this mini-review, we will first discuss recent imaging-based findings from our group supporting the existence of major differences in endocytosis among PT segments, and showing that S1 is highly specialized for protein uptake. We will then summarize evidence from other studies suggesting that this situation might change in disease states, where compensatory uptake in S2 and S3 segments could serve to minimize protein loss in the urine. Finally, we will briefly consider emerging evidence from *in vitro* studies regarding important environmental factors that can regulate endocytosis, which might explain how tubular uptake capacity is matched to the filtered load in the functioning kidney.

New imaging studies of endocytosis in the proximal tubule

Numerous older studies in rats and rabbits reported an axial decline along the PT in the expression and activity of lysosomal enzymes and in apical membrane bound amino-peptidases (23, 25, 35, 46–48). This suggests, somewhat intuitively, that the early PT is predominantly responsible for protein uptake and degradation. However, whether or not there is a step-change in endo-lysosomal function between S1 and S2 segments remained unclear, especially in the mouse.

To address this question, we injected fluorescently labeled ligands for endocytosis into mice and followed their uptake into PTs in real-time using intravital multiphoton microscopy (41). We observed that uptake of small proteins occurs almost exclusively in S1 segments, even when injected in amounts way beyond normal physiological concentrations (**Figure 1A**). Moreover, antibody staining in fixed tissue for endogenous protein ligands also revealed uptake exclusively in early S1 segments. In contrast, uptake of dextrans by fluid-phase endocytosis was much less efficient in S1, and substantial reabsorption was also observed in S2 segments, along with wasting in distal tubules (**Figure 1B-C**). Meanwhile, electron microscopy revealed that even in the mouse ultrastructural differences are evident between S1 and S2 cells, with large apical vacuoles only occurring in the former. Furthermore, at both the RNA and protein level we found evidence that expression levels of key endo-lysosomal markers display a bimodal distribution, being much higher in S1 than S2.

One major constraint of intravital kidney imaging is the limited depth of imaging possible (40). To circumvent this problem, following uptake of ligands *in vivo* we fixed and removed kidneys, then subjected them to a tissue clearing process. We used a modified CLARITY protocol that increased the transparency of the tissue, whilst retaining the fluorescence signals from internalized ligands. We were subsequently able to image large tissue sections in 3-D (**Figure 1D**), and trace uptake lengths along individual nephrons. This confirmed that uptake patterns of protein and dextran are markedly different throughout the entire cortex. Moreover, we observed a relatively sharp cut-off point between protein and dextran uptake, probably corresponding to the interface of early (S1) and late (S2) PT segments.

Taken together, these findings support the existence of two discrete functional segments in the mouse PT with regards to endocytosis. Moreover, they suggest that the S1 segment is highly specialized to perform protein reabsorption, and has a very large uptake capacity (23). Although we are not aware of any attempts to specifically quantify axial patterns of protein uptake in the rat PT, we note that previous imaging studies have also emphasized a high S1 uptake (8). Conversely, endocytosis of lysozyme was reported to be low in isolated rabbit S2 segments (32). Thus, it is likely that axial differences in PT endocytosis also exist in other species, although this requires further detailed investigation.

These observations have potentially important clinical implications. For example, they could help to explain topographical patterns of damage along the PT resulting from toxins that are ligands for either receptor-mediated or fluid-phase endocytosis, such as light chains, aminoglycoside antibiotics, contrast agents and volume expanders (7, 15, 17, 22). Moreover, the high uptake capacity suggests that the appearance of small proteins in the urine is probably not a very sensitive marker of S1 damage.

Given that the uptake of filtered substances – including proteins – seems to occur predominantly in S1, one question logically arising is whether S2 segments have other specialized functions. Organic cations and anions, such as drugs or their metabolites, are eliminated in large quantities from the blood to the urine through active transcellular excretion

in the PT, involving a series of basolateral and apical membrane transporters (45). Expression levels of anion/cation transporters is generally greater in more distal PT segments, and previous experiments in isolated rabbit PTs have reported very high levels of organic anion secretion in S2 segments (30, 38, 39, 42). Using intravital imaging, we also found that a substrate for organic-anion transporter 1, monochlorobimane, showed a higher uptake in S2 than in S1 (41). However, further experiments will be required to fully elucidate the specific functions of S2 PT segments in the mouse.

Development and plasticity of endocytosis along the proximal tubule

Previous electron microscopy studies of rat kidneys have shown that the full development of the endo-lysosomal system in PT cells is reached only in the late stages of gestation, following the onset of glomerular filtration (19). The full differentiation of S1 and S2 segments then occurs over the first weeks post-birth in rats and in dog pups (16, 19). These observations suggest that axial patterns in endocytotic capacity along the PT are predominantly shaped by environmental factors, most likely related in some manner to the filtered load of proteins.

In support of this notion, experimental occlusion of early S1 segments in rats leads to a de-differentiation of downstream PT cells (44). Conversely, increasing glomerular proteinuria by acute treatment with adriamycin or puromycin aminonucleoside increases lysosomal protein expression and activity in more distal PT segments (9) (34). Moreover, lysosomal cathepsin activity in the S2 and S3 segments of Munich Wistar Frömter rats, which display a physiological proteinuria, is higher than in the late PT of normoproteinuric Sprague-Dawley rats (9). Finally, recent studies have shown that genetic ablation in mice of the endocytic receptor megalin in S1 and S2 PT segments induces an increase in protein uptake in S3 (31).

Taken together, these studies suggest that in times of increased protein delivery, the capacity of the distal PT segments (S2 and S3) to endocytose and degrade proteins can increase, presumably as a compensatory response to minimize wasting in the urine. Of note,

we observed a similar expression level of megalin in both S1 and S2 segments in mice (41), implying that the latter has the capability to bind luminal proteins, and thus perhaps to "sense" when their concentration is increased. Although the exact environmental factors that stimulate and drive increased endocytotic capacity in PT cells *in vivo* remain to be elucidated, some potentially important new insights have recently been obtained from elegant studies performed with *in vitro* cell models.

Mechanisms driving tubular endocytic activity

In a series of studies using cultured opossum kidney cells (one of the very few immortalized PT-derived lines that retain significant amounts of endocytosis) Weisz and colleagues have elucidated some of the important steps that appear to link environmental cues with cellular differentiation and protein uptake capacity (for detailed reviews please see (10, 36)). They have found that apical fluid shear stress induces rearrangements in the endo-lysosomal system and promotes endocytosis. Mechanistically, this is explained by bending of primary cilia, release of ATP, autocrine/paracrine purinergic signaling and increases in intracellular calcium. Such rises in intracellular calcium might promote apical endocytosis through calcium-calmodulin binding and Cdc42 activation (1). The master regulator mTOR, which has a very close functional relationship with the endo-lysosomal system, has also been identified as a key player (21), and mice deficient in mTOR in the PT display a severe defect in endocytosis (12). Finally, very recent studies have revealed that increased oxygen availability also drives differentiation in opossum kidney cells (37), thus representing a potential link between metabolism and endocytosis. In summary, these *in vitro* observations provide pointers to the key processes that might upregulate endocytosis in PTs during disease states and compensatory remodeling, which can now be further explored *in vivo*.

Summary and future perspectives

A variety of different lines of evidence suggest that there are major axial differences in endocytosis along the PT, including in the mouse. The early S1 segment is highly adapted to perform receptor mediated endocytosis and has a very large protein uptake capacity.

Therefore, under normal physiological conditions uptake of filtered proteins occurs almost exclusively in this region. However, when distal protein delivery is increased, due to either increased glomerular filtration and/or an uptake defect in S1, it seems that S2 and S3 PT segments can increase uptake and degradation capacity, to minimize protein loss in the urine. The PT can therefore be considered as a plastic epithelium, undergoing morphological and functional adaptations in response to micro-environmental stimuli. Recent *in vitro* studies have provided plausible explanations regarding the underlying mechanisms, but these are yet to be confirmed *in vivo*. Moreover, it remains unclear whether compensatory increases in endocytosis in distal PT segments could have deleterious effects, such as an accompanying decrease in normal S2 and S3 secretory functions. Thus, there is much still to learn about the basic physiology of the PT in living animals, but intravital microscopy represents a potentially powerful tool to fill the many gaps in our knowledge.

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Author contributions

M.P. and A.M.H. prepared, edited, revised and approved the final version of the manuscript.

Disclosures

None.

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Figure legends

Figure 1: Intravital multiphoton microscopy reveals axial differences in endocytosis along the mouse proximal tubule. (**A**) Lysozyme, a protein substrate for receptor mediated endocytosis, is predominantly reabsorbed in S1 (#), whilst no S2 (*) uptake occurs and very little wasting can be detected in the distal nephron (arrowhead). (**B**) In contrast, dextran, a marker for fluid phase endocytosis, is taken up in both S1 and S2 segments, and substantial wasting in the distal nephron is observed. (**C**) Overlay showing lysozyme (red) and dextran (green) uptake patterns. (**D**) 3-D image of a large section of cleared mouse kidney cortex from the capsule (top-right) to the medulla (bottom-left), showing the uptake of lysozyme in proximal tubules. Scale bars: 100μm in A-C; 0.5mm in D.